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Epimerization-free synthesis of cyclic peptide by use of the *O*-acyl isopeptide method

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A head-to-tail cyclization of a protected linear hexapeptide with a *C*-terminal *O*-acyl isopeptide proceeded to give a cyclic *O*-acyl isopeptide without epimerization. The cyclic *O*-acyl isopeptide possessed different secondary structures compared with the native cyclic peptide. The isopeptide was then efficiently converted to the desired cyclic peptide via an *O*-to-*N* acyl migration reaction using a silica gel-anchored base. Copyright © 2010 European Peptide Society and John Wiley & Sons, Ltd.

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Keywords: racemization; cyclic peptide; isopeptide; epimerization; *O*-to-*N* acyl migration

Introduction

Cyclic peptides often possess higher biological activity than their corresponding linear peptides due to conformational constraints and less entropic penalty [1–4]. Additionally, the rigid structure of the cyclic peptides generally increases metabolic resistance against biodegradations [5]. Despite the promises of cyclic peptides in drug development, a head-to-tail cyclization reaction of a corresponding linear peptide is often accompanied by incomplete coupling and/or epimerization (Figure 1A) [6,7]. The epimerization occurs because, in contrast to urethane-protected amino acids, peptides easily form chirally labile oxazolones upon *C*-terminal carboxyl activation.

We discovered that the presence of an *O*-acyl instead of *N* acyl residue within the peptide backbone significantly changed the secondary structure of the native peptide. In addition, the target peptide was subsequently generated by an *O*-to-*N* intramolecular acyl migration reaction. These findings led to the development of a novel method, called '*O*-acyl isopeptide method', for peptide synthesis [8,9]. To date, the *O*-acyl isopeptide method has also been used in various research fields [10–21]. Along these lines, we [22,23] and Coin *et al*. [24] previously reported a novel convergent method of peptide synthesis: racemization-free segment condensation based on the *O*-acyl isopeptide method (Figure 1B). The idea was that an *N*-segment with a *C*-terminal *O*-acyl isopeptide structure at Ser or Thr residue could be coupled to an amino group of the *C*-segment without epimerization, because the amino group of the *C*-terminal isopeptide is protected by a urethane-type protective group. Thus, formation of the epimerization-inducible oxazolone would be suppressed upon carboxyl group activation. Finally, the deprotected *O*-acyl isopeptide released the native peptide via the *O*-to-*N* acyl migration reaction.

If the racemization-free segment condensation strategy is applied to an intramolecular system, a linear peptide with a *C*-terminal *O*-acyl isopeptide could undergo epimerization-free cyclization due to the urethane-type protective group at the Ser/Thr residue, and following the *O*-to-*N* acyl migration of the cyclic *O*-acyl isopeptide, would give the target cyclic peptide (Figure 1C). Martinez and Amblard's group reported a proof-of-principle by constructing cyclic peptides via *O*-acyl isopeptides [25]. In the communication, the *O*-acyl isoforms of cyclic tetra, penta, hexa and heptapeptides were successfully synthesized. They also showed that the subsequent *O*-to-*N* acyl migration reaction effectively proceeded to afford cyclic penta, hexa and heptapeptides, while the formation of cyclic tetrapeptide was difficult probably due to the constraint of the final structure. We herein report (i) a comparative study on epimerization extent in head-to-tail cyclization reactions of the native peptide and *O*-acyl isopeptide, (ii) optimal reaction conditions to form the cyclic *O*-acyl isopeptide, (iii) a concise *O*-to-*N* intramolecular acyl migration using a silica gel-anchored base and (iv) the secondary structure of the cyclic *O*-acyl isopeptide.

Results and Discussion

Cyclization Reaction of a Hexapeptide by a Conventional Solution Phase Method

The cyclization of protected linear hexapeptide **1** (H-Arg(Pmc)-Ala-Gly-Asn(Trt)-Ala-Ser(tBu)-OH, for synthesis: see Section on Experimental) was performed under three conditions (Table 1): HATU–collidine method [26–28] in DMF (Entry 1), HATU–collidine method in CH_2Cl_2 (1% DMF contained, Entry 2) and DPPA (diphenylphosphoryl azide)–$NaHCO_3$ [29,30] method in DMF

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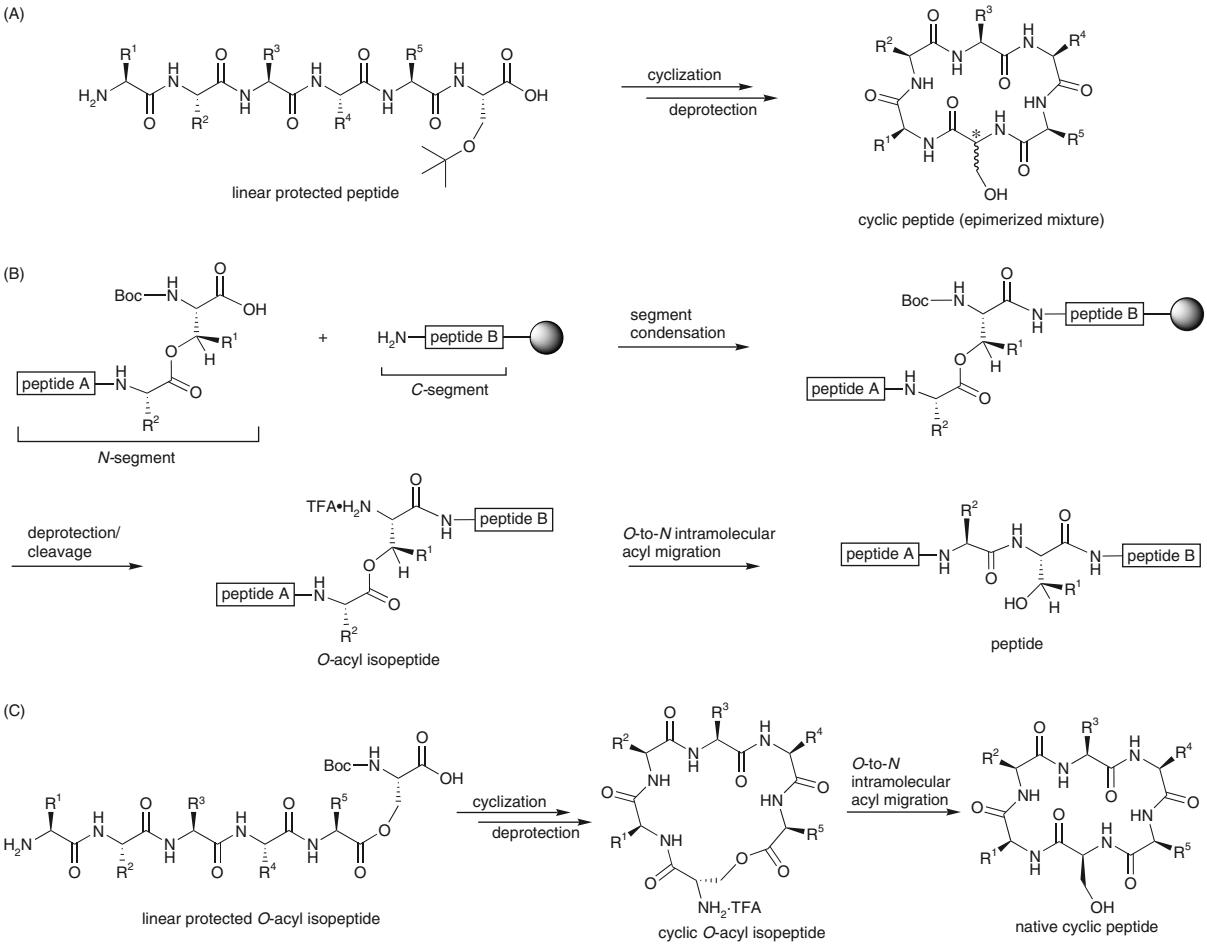

Figure 1. (A) A conventional synthesis of cyclic peptide via a head-to-tail cyclization of a linear peptide; (B) segment condensation methodology based on the *O*-acyl isopeptide method; (C) synthesis of cyclic peptide by use of the *O*-acyl isopeptide method.

(Entry 3). In Entry 1 condition, 39% (HPLC yield) of the cyclic [D-Ser] derivative was observed in addition to the desired **2** (60%) after 12 h reaction (Figure S1, Supporting Information), indicating that a large amount of epimerization occurred during the cyclization reaction. When CH_2Cl_2 was used (Entry 2), the [D-Ser] derivative was 2%. In Entry 3, 21% of the starting material **1** remained even after 5 days and the [D-Ser] derivative (15%) was also observed. The epimers observed in Entries 1–3 were probably due to the formation of the chirally labile oxazolones upon *C*-terminal carboxyl activation.

Cyclization Reaction in the *O*-Acyl Isopeptide

As shown in Table 2, a protected *O*-acyl isopeptide (**3**, Boc-Ser(H-Arg(Pmc)-Ala-Gly-Asn(Trt)-Ala)-OH), for synthesis: see Section on Experimental; crude HPLC: Figure S2, Supporting Information) was cyclized under Entries 1–3 conditions. In the HATU–collidine method in DMF (Entry 1), the desired **4** (94%, HPLC yield) was obtained after 12 h reaction and the D-Ser derivative derived from epimerization was not detected (detection limit: 0.5%) in the crude sample (Figure 2A). This result indicates that the [D-Ser] formation (39% in the cyclization of the corresponding native peptide by the conventional method) could be suppressed in the cyclization at the Boc-protected *O*-acyl isopeptide, suggesting that the ring-closing reaction did not involve the formation of oxazolone. In Entry 2 with CH_2Cl_2 (1% DMF contained), 94% of **4** was formed after 1 h reaction without epimerization (Figure 2B). In the cyclization with DPPA (Entry 3), the des-Ser by-product (36%) was detected in addition to the desired **4**, and a few percent of a peak derived from the D-Ser derivative was detected (Figure 2C). The des-Ser by-product would be derived from the formation of an intramolecular mixed anhydride with Boc-ΔAla-OH upon activation of the carboxyl group (Scheme S1, Supporting Information), as proposed in our previous study with the *O*-acyl isodipeptide unit [31]. Finally, the isolated yield of cyclic *O*-acyl isopeptide **5**·TFA after two steps from **3** (i.e. cyclization of **3** by Entry 2 + deprotection of **4** with TFA-cocktail followed by HPLC purification) was 53% (Scheme 1, Figure 3A).

O-to-*N* Intramolecular Acyl Migration Reaction of Cyclic *O*-Acyl Isopeptide to Afford Cyclic Hexapeptide

In neutral phosphate buffer (pH 7.6), the isopeptide **5**·TFA was converted to the native cyclic peptide **6** via the *O*-to-*N* intramolecular acyl migration reaction (Figure S3, Supporting Information). The reaction was completed within 1 min. The result suggests that the *O*-to-*N* acyl migration reaction could rapidly proceed in conformationally constrained cyclic isopeptide as well as linear peptides, which agrees with the results from Martinez and Amblard's group [25] and a tendency for the *O*-to-*N* acyl shift to take place during the synthesis of cyclic depsipeptide [32]. We next attempted the rearrangement reaction in the presence of a silica

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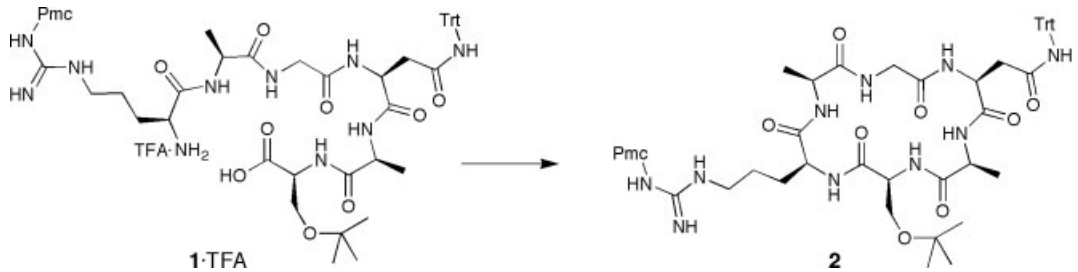
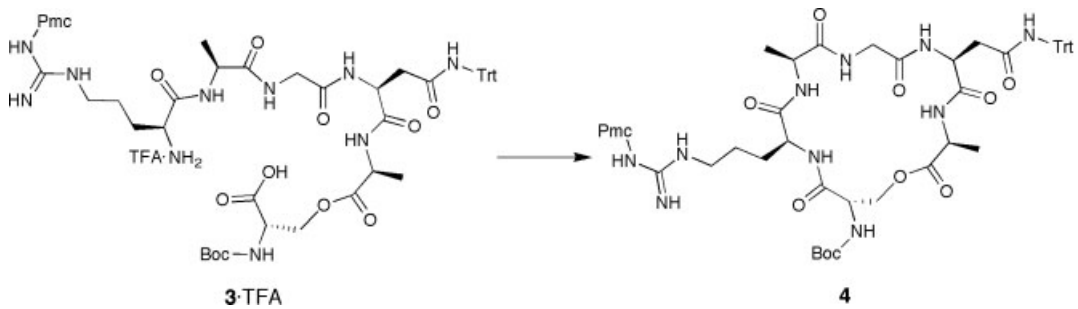
Table 1. Cyclization of protected linear peptide **1** TFA[a]



Entry	Reagents	Solvent	Time	Yield[b] (%) **1** : **2**:[D-Ser]-**2**[c]
1	HATU (2 eq)-collidine (4 eq)	DMF	12 h	1 : 60 : 39
2	HATU (2 eq)-collidine (4 eq)	CH_2Cl_2[d]	1 h	3 : 95 : 2
3	DPPA (3 eq)-$NaHCO_3$ (3 eq)	DMF	5 days	21 : 64 : 15

[a] Peptide concentration = 1 mM; temp = rt.
[b] Yields were estimated by the peak area in analytical HPLC.
[c] Authentic D-Ser derivative was synthesized independently and used to determine the ratio of epimerization by HPLC.
[d] Containing 1% DMF.

Table 2. Cyclization of protected *O*-acyl isopeptide **3** TFA[a]



Entry	Reagents	Solvent	Time	Yield[b] (%) **3** : **4**:[D-Ser]-**4**[c]:des-Ser-**4**[d]
1	HATU (2 eq)-collidine (4 eq)	DMF	12 h	6 : 94 : N.D.[e] : N.D.
2	HATU (2 eq)-collidine (4 eq)	CH_2Cl_2[f]	1 h	6 : 94 : N.D. : N.D.
3	DPPA (3 eq)-$NaHCO_3$ (3 eq)	DMF	5 days	1 : 61 : 2 : 36

[a] Peptide concentration = 1 mM; temp = rt.
[b] Yields were estimated by the peak area in analytical HPLC.
[c] Authentic D-Ser derivative was synthesized independently and used to determine the ratio of epimerization by RP-HPLC.
[d] Cyclo(RAGNA) + the [D-Ala] derivative.
[e] N.D., not detected (detection limit: 0.5%).
[f] Containing 1% DMF.

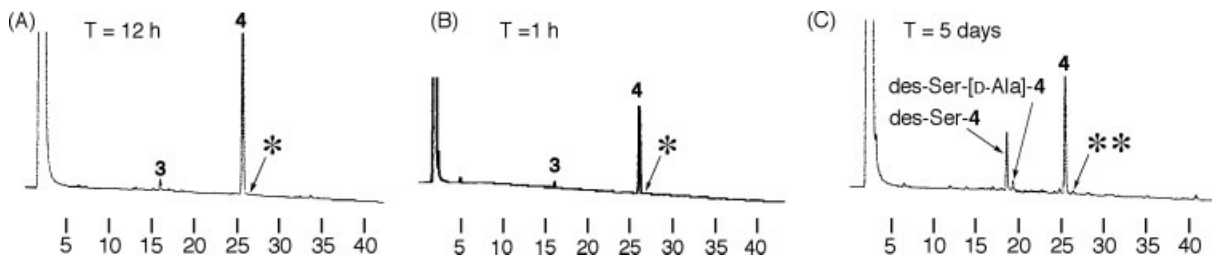
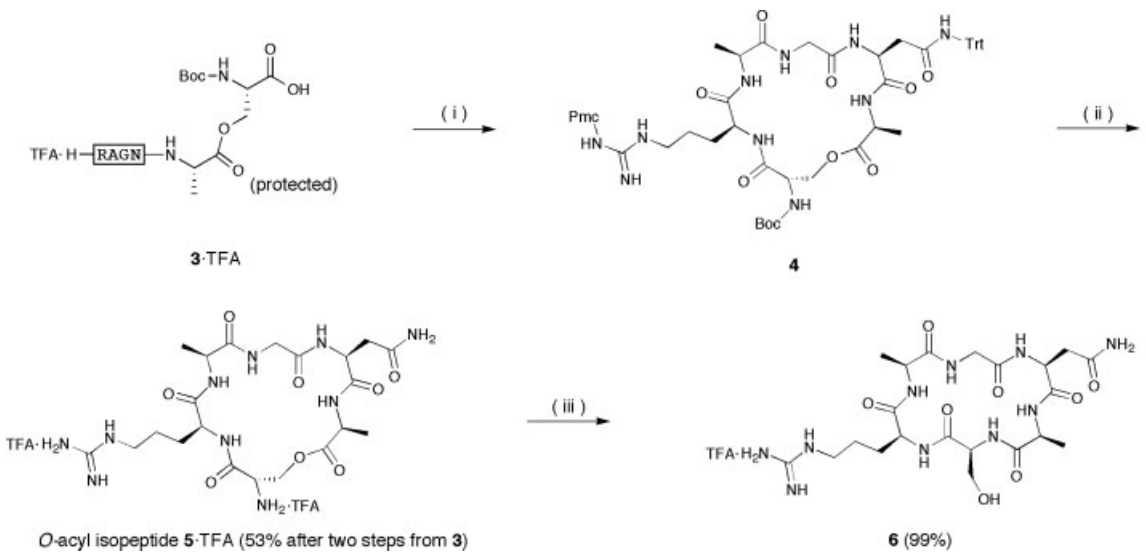


Figure 2. HPLC charts of crude **4** from**3** in Entry 1 (A), Entry 2 (B) and Entry 3 (C). *No detectable peak derived from [D-Ser]-**4** (detection limit: 0.5%). **~2% of a peak derived from the D-Ser derivative was detected. Analytical HPLC was performed using a C18 reverse phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN (45–100% CH_3CN, 55 min) in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 230 nm.



Scheme 1. Reagents and conditions: (i) HATU (2 eq), 2,4,6-collidine (4 eq), CH_2Cl_2 (containing 6% DMF), final peptide conc: 1 mM, 3 h, rt; (ii) TFA (92.5%)-*m*-cresol (2.5%)-thioanisole (2.5%)-H_2O (2.5%), 60 min, rt; (iii) 3-(trimethylammonium)propyl-functionalized silica gel carbonate (2 eq), CH_3CN/H_2O (1 : 1), 3 h, rt, then treated with 0.1% aqueous TFA.

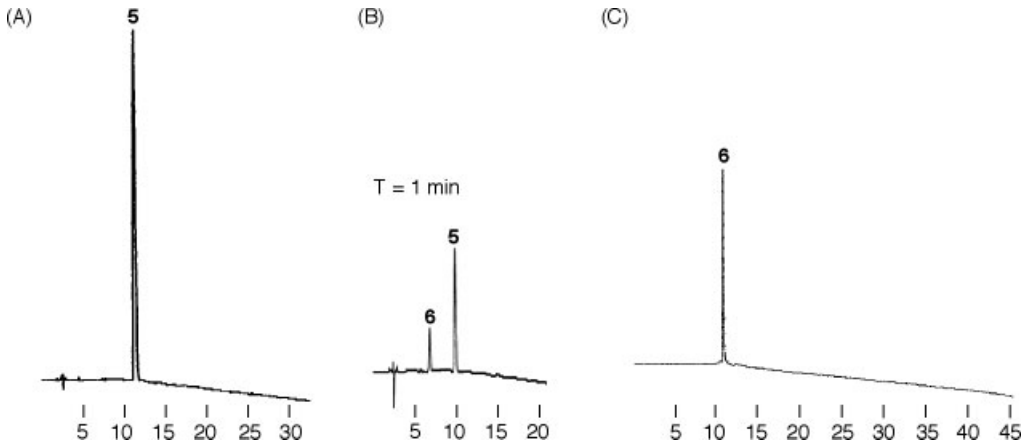


Figure 3. (A) Purified cyclic *O*-acyl isopeptide **5**; (B) *O*-to-*N* acyl migration of **5** to give **6** in water/CH_3CN (1 : 1) in the presence of silica gel-anchored base (2× excess mole), $T = 1$ min; (C) pure cyclic peptide **6**, synthesized via the isopeptide **5**. Analytical HPLC was performed using a C18 reverse phase column (4.6 mm × 150 mm; YMC Pack ODS AM302) with binary solvent system: a linear gradient of CH_3CN [2–22% CH_3CN, 40 min for (A, B); 0–100% CH_3CN, 40 min for (C)] in 0.1% aqueous TFA at a flow rate of 0.9 ml min^{-1} (40 °C), detected at 220 nm for (A, B) and 230 nm for (C).

gel-anchored base, 3-(trimethylammonium)propyl-functionalized silica gel carbonate. With the silica gel-anchored base, the desired peptide can readily be separated by a simple filtration. When the isopeptide **5**·TFA was dissolved in water/CH_3CN (1 : 1) in the presence of two molar excess of silica gel-base, approximately 25% of **6** was formed within 1 min (Figure 3B) and a quantitative *O*-to-*N* acyl migration was observed after 3 h reaction without any side reaction. The pure target peptide **6** was isolated after filtration followed by lyophilization of the filtrate (Figure 3C). When the same reaction was performed in water (without acetonitrile), ~2% of hydrolyzed product was observed at the ester of **5**, although the *O*-to-*N* migration was completed within 1 h. The obtained **6** had identical elution time on the HPLC analysis as the authentic sample prepared by the conventional synthetic method.

Secondary Structure of Cyclic *O*-Acyl Isopeptide

Secondary structures of **5** and **6** in pH 2.6 phosphate buffer containing 38 mM NaCl at room temperature were examined by CD spectrometry (Figure 4). In the CD spectrum of **5**, a negative maximum at 190 nm and positive ellipticity around 200 nm were observed. In the case of the corresponding native peptide **6**, a positive ellipticity around 190 nm and negative maximum around 203 nm were observed. These results indicated that the *O*-acyl isopeptide **5** and cyclic peptide **6** adopted different secondary structures, suggesting that a well-known secondary structure disrupting effect by the introduced *O*-acyl isopeptide observed in many linear peptides [8–21] is also exerted in cyclic peptides.

Experimental

*H-Arg(Pmc)-Ala-Gly-Asn(Trt)-Ala-Ser(tBu)-OH (**1**)*

Protected linear peptide **1**·TFA was synthesized in a similar manner to that described in Ref. 23 (0.088 mmol scale). Yield: 42.3 mg, 0.034 mmol, 38% (based on the resin-bound-Ser). MALDI-MS (TOF):

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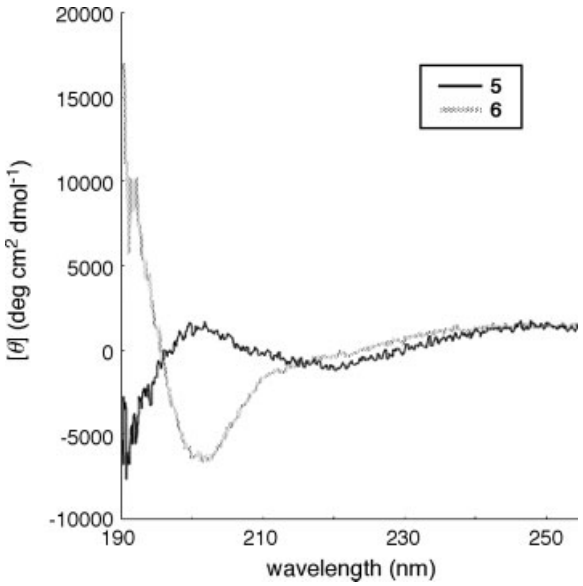

Figure 4. Circular dichroism (CD) spectroscopy of the *O*-acyl isopeptide **5** and the corresponding native peptide **6** (0.1 mM each) in pH 2.6 phosphate buffer containing 38 mM NaCl, measured at room temperature. The samples were measured right after dissolving in the buffer. The molar ellipticity [θ] values have been normalized for the number of backbone amide groups.

M_{calc}: 1139.4; M + Na_{found}: 1162.3; HPLC analysis at 230 nm: purity was 93%.

*Boc-Ser(H-Arg(Pmc)-Ala-Gly-Asn(Trt)-Ala)-OH (**3**)*

Protected *O*-acyl isopeptide **3**·TFA was synthesized in a similar manner to that described in Ref. 23 (0.046 mmol scale). Yield: 27.2 mg, 0.021 mmol, 46% (based on the resin-bound-isodipeptide unit). MALDI-MS (TOF): M_{calc}: 1183.4; M + Na_{found}: 1206.0. HPLC analysis at 230 nm: purity was 97%.

*O-acyl isopeptide of cyclo(Arg-Ala-Gly-Asn-Ala-Ser) (**5**)*

To a stirring solution of **3**·TFA (46.7 mg, 0.036 mmol) in DCM (36 ml, containing 6% DMF), HATU (27.4 mg, 0.072 mmol) and 2,4,6-collidine (19.1 µl, 0.14 mmol) were added, and the mixture was stirred for 3 h at room temperature. The reaction mixture was condensed *in vacuo* to afford the protected cyclic isopeptide **4**. MALDI-MS (TOF): M_{calc}: 1165.4; M + Na_{found}: 1188.0. The crude **4** was treated with TFA (2.79 ml)–*m*-cresol (75 µl)–thioanisole (75 µl)–water (75 µl) (92.5 : 2.5 : 2.5 : 2.5) for 45 min at room temperature, and then concentrated *in vacuo*. After triturating with diethyl ether, the resulting white precipitate was dissolved in water (1.5 ml) and immediately purified by preparative RP-HPLC with a 0.1% aqueous TFA–CH_3CN system. The desired fractions were collected and immediately lyophilized to afford the desired cyclic *O*-acyl isopeptide **5**·TFA as a white amorphous powder. Yield: 15.1 mg, 0.019 mmol, 53%; MALDI-MS (TOF): M_{calc}: 556.6; M + H_{found}: 557.6; HPLC analysis at 230 nm: purity was 95%.

O-acyl isopeptide of cyclo(Arg(Pmc)-Ala-Gly-Asn(Trt)-Ala-D-Ser)

The [D-Ser] form of the protected cyclic isopeptide was synthesized in a similar manner to **4**. MALDI-MS (TOF): M_{calc}: 1165.4; M + Na_{found}: 1188.7.

*Cyclo(Arg-Ala-Gly-Asn-Ala-Ser) (**6**)*

To a solution of **5**·TFA (6.1 mg, 7.8 µmol) in water–acetonitrile (1 : 1, 0.2 ml), was added 3-(trimethylammonium)propyl-functionalized silica gel carbonate (21.7 mg, 15.6 µmol) (final pH 7). After the solution was shaken for 3 h at room temperature, the silica gel was filtered off and the desired peptide was washed out with 4.8 ml of water containing 0.1% v/v TFA. The filtrate was lyophilized to give **6**·TFA as a white amorphous powder. Yield: 5.2 mg, 7.8 µmol, >99%; MALDI-MS (TOF): M_{calc}: 556.6; M + H_{found}: 557.6; HPLC analysis at 230 nm: purity was 96%.

Conclusions

We herein showed that a cyclic hexapeptide was synthesized in an epimerization-free fashion using the *O*-acyl isopeptide method. The head-to-tail cyclization of the linear peptide with a *C*-terminal *O*-acyl isopeptide proceeded to give the cyclic *O*-acyl isopeptide without epimerization. Interestingly, the cyclic *O*-acyl isopeptide possessed a different secondary structure when compared with the native cyclic peptide. Finally, the isopeptide was efficiently converted to the desired cyclic peptide via the *O*-to-*N* acyl migration reaction.

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Supporting information

Supporting information may be found in the online version of this article.

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